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26 JUL 2002

3. Full name of each applicant *(underline all surnames)*

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Patents ADP number *(if you know it)*

If the applicant is a corporate body, give the country/state of its incorporation

7986780001

5677729001

4. Title of the invention

Novel Albumins

5. Name of your agent *(if you have one)*

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

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Patents ADP number *(if you know it)* 547002

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NOVEL ALBUMINS

Human albumin is the most abundant protein in blood plasma. Typically, it is present at concentrations of around 750 μM. It is a single polypeptide chain of 585 amino acids with a largely helical triple-domain structure. The gene for human serum albumin comprises 16,961 nucleotides from the supposed "capping" site up to the first site for addition of poly(A).

Albumin is the major transport protein in the blood and can reversibly bind to a wide range of small molecules, such as fatty acids, hormones, and drugs. Albumin is also implicated in the transport and storage of many metal ions. Presently, human albumin is used clinically in the treatment of patients with severe burns, shock or blood loss. Other mammalian albumins are highly homologous with human albumin.

Zinc and copper are known to bind albumin with association constants of 3.4×10^7 and 1.5×10^{16} M^{-1} respectively (Masuoka *et al.* (1993) J. Biol. Chem. 268, 21533-21537). Cu^{2+} binds most strongly to albumin's N-terminal amino acids Asp1-Ala2-His3, which provide a square-planar site of 4 N ligands, although other binding sites on the molecule are known to exist.

Approximately 75% of Zn^{2+} in blood plasma (*ca.* 14 μM) is bound to albumin. This accounts for as much as 98% of the exchangeable fraction of Zn^{2+} in serum (Giroux *et al.* (1976) J. Bioinorg. Chem. 5, 211-218; Foote and Delves (1984) *Analyst* 109, 709-711). Albumin has previously been shown to modulate zinc uptake by endothelial cells, whilst

receptor-mediated vesicular co-transport across the endothelium has been demonstrated with albumin-zinc complexes *in vitro* (Bobilya *et al.*(1993) Proc. Soc. Exp. Biol. Med. 202, 159-166; Tibaduiza *et al.*(1996) J. Cell. Physiol. 167, 539-547). No binding sites for Zn^{2+} on albumin had previously been specifically located, even though albumin is believed to be the main zinc transport protein in the circulation.

Zinc is an essential element in the body and is present in over 300 enzymes. It has many important roles including the transport of vitamin A, the healing of wounds, sperm production in men and is recruited by anthrax lethal factor and bacterial enterotoxin. The regulation of zinc levels in the blood is therefore physiologically very important. It has been proposed that Zn^{2+} recruitment from blood can be used to increase the affinity of certain metal-binding organic drugs for proteins and enzymes, e.g. benzimidazole inhibitors of serine proteases such as trypsin (Katz and Luong (1999) J. Mol. Biol. 292, 669-684; Janc *et al.* (2000) Biochemistry 39, 4792-4800; Katz *et al.*(2001) Chem. & Biol. 8, 1107-1121; Liang *et al.* (2002) J. Am. Chem. Soc. *in press*).

The present invention is based on the discovery that a cluster of four amino acids (His67, Asn99, His247 and Asp249), which lie at the interface between domains I and II are involved in a binding site for zinc, copper and/or cadmium (see Figures 1 and 2). All four of these residues are highly conserved amongst all mammalian albumins sequenced to date (see Table 1). The numbering referred to herein relates to the amino acid found at the particular position of the human serum albumin amino acid sequence after the pre-albumin sequence has been cleared following translation (see Table 1). Identification of this site provides a rational for the design of therapeutic albumins for controlling the

levels of available zinc and/or other metal ions in blood and their delivery to target tissues.

Thus, in a first aspect there is provided a mutant human serum albumin comprising the sequence:

DAHKSEVAHRFKDLGEENFKALVLIAFAQX_5LQQCPFEDHVKL

VNEVTEFAKTCVADESAENCDKSLX_1TLFGDKLCTVATLRETY

GEMADCCAKQEPERX_2ECFX_6QHKDDNPNLPRLVRPEVDVMCT

AFHDNEETFLKKYLYEIARRHPYFYAPELLFFAKRYKAAFTEC

CQAADKAACLLPKLDELRDEGKASSAKQRLKCASLQKFGERA

FKAWAVARLSQRFPKAEFAEVSKLVTDLTKVHTECC$X_3X_7X_4$LL

ECADDRADLAKYICENQDSISSKLKECCEKPLLEKSHCIAEVEN

DEMPADLPSLAADFVESKDVCKNYAEAKDVFLGMFLYEYARR

HPDYSVVLLLRLAKTYETTLEKCCAAADPHECYAKVFDEFKPL

VEEPQNLIKQNCELFEQLGEYKFQNALLVRYTKKVPQVSTPTL

VEVSRNLGKVGSKCCKHPEAKRMPCAEDYLSVVLNQLCVLHE

KTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFNAETFTF

HADICTLSEKERQIKKQTALVELVKHKPKATKEQLKAVMDDF

AAFVEKCCKADDKETCFAEEGKKLVAASQAALGL

wherein X_1, is other than H; X_2 is other than N, X_3 is other than H, X_4 is other than D; X_5 is other than Y; X_6 is other than L and/or X_7 is other than T, such that said mutant displays an altered metal binding affinity with respect to native human serum albumin.

The above sequence is based on the human form of serum albumin after a leader sequence (ie. MKWVTFISLLFLFSSAYSRGVFRR) has been cleaved from the sequence. The present invention also extends to mutant sequences including such leader sequences.

While the above relates to mutants of human serum albumin, it is to be understood that the present invention is not limited to only mutant human serum albumins. Serum albumins across all species display a high degree of conservation and it is well within the expertise of the skilled addressee to identify the amino acids in the positions represented by Xs in the sequence above, from albumins of other species and change said amino acids in order to alter metal binding. Table 1 in fact shows an alignment of mammalian serum albumin polypeptide sequences in which the residues which may change, are highlighted. It is understood that at least one of said residues should be other than the identified native residue in order to generate a mutant serum albumin, which may display altered metal binding with respect to the native species serum albumin.

The sequences of many serum albumin are known and readily available from the Genbank database at, for example, the National Center for Biotechnology Information: www.ncbi.nlm.nih.gov. The human sequence may for example be found under accession number P02768. Other accession numbers may also be found at www.albumin.org.

It is understood that the mutants of the present invention should be substantially similar in terms of general overall folding with respect to the native serum albumin of the particular species. For example circular dichroism studies may be conducted to see

whether or not signs and magnitudes of cicular dichroism bands of a mutant serum albumin are similar to native serum albumin. If they are similar this would be indicative of the mutant serum albumin displaying similar secondary structure to the native serum albumin.

The mutants of the present invention should display an altered metal binding affinity with respect to the native albumin from which the mutant is derived. Altered metal binding affinity is understood to mean a decrease or increase in metal binding affinity (e.g. Km) and/or an increase or decrease in the rate of binding/dissociation of the metal. The metals, which may display altered binding affinity to such mutant albumins, are zinc, copper, nickel and cobalt. Preferably the mutant albumins display altered binding affinity for zinc. Generally, the altered metal binding affinity will be with respect to a metal ion, such as Zn^{2+}, Cu^{2+}, etc. Mutation of residues thought to be involved with metal binding to residues which possess appropriate metal binding side chains are postulated to result in decreased metal binding affinity. Conversely mutation of residues not involved in metal/metal ion binding, but which are in the vicinity of the residues which are thought to be involved in binding to metal, to residues which assist/facilitate binding, would be expected to increase metal binding affinity.

For example, the following mutations are postulated to result in decreased metal binding affinity:

$X_1 \Rightarrow$ A, D, E, F, G, I, K, L, N, P, Q, R, S, T, V, W, Y

$X_2 \Rightarrow$ A, F, G, I, K, L, P, Q, R, S, T, V, W, Y

$X_3 \Rightarrow$ A, D, E , F, G, I, K, L, N, P, Q, R, S, T, V, W, Y

$X_4 \Rightarrow$ A, F, G, I, K, L, N, P, Q, R, S, T, V, W, Y

and mutation of a side-chain to introduce a metal binding ligand that is likely to give rise to increased metal affinity include:

$X_5 \Rightarrow$ C, D, E, H (this is already a His residue in pig albumin)

$X_2 \Rightarrow$ C, D, E, H

$X_6 \Rightarrow$ C, D, E, H

$X_7 \Rightarrow$ C, D, E, H

$X_4 \Rightarrow$ C, E, H

$X_1 \Rightarrow$ D, E

$X_3 \Rightarrow$ D, E

It should be appreciated that standard one-letter amino acid nomenclature is used throughout this description.

The nature of the Zn^{2+} binding site on albumin was indicated by ^{113}Cd-NMR studies. Several mammalian albumins have two strong binding sites for Cd^{2+} with chemical shifts characteristic of N/O coordination (Sadler and Viles (1996) Inorg. Chem. 35, 4490-4496). For human albumin, ^{113}Cd shifts of 24 and 114 ppm (relative to $Cd(ClO_4)$) are indicative of sites containing a single imidazole nitrogen and 2-3 imidazole nitrogens, respectively. Zn^{2+}, Cu^{2+} and Ni^{2+} ions can displace Cd^{2+} from the latter of these sites in human albumin. The present inventors' molecular modeling based on the crystal structure of albumin (PDB 1AO6) suggested that the multi-metal binding site might involve the cluster His67, Asn99, His247 and Asp249. The present inventors established the location of this site through site-directed mutagenesis of His67 to alanine followed by metal competition studies with isotopically enriched cadmium using ^{111}Cd NMR.

Conventionally this may be represented as H67A, which identifies the histidine at position 67 being mutated to alanine. Such representation, is used elsewhere in the description.

The mutated albumins of the present invention may be synthesized de novo, but preferably they are produced by recombinant means well known to those skilled in the art. The mutated albumins can, for example, be derived from the native albumin by carrying out site-directed mutagenesis on the associated gene sequence and subsequent expression of the protein. Such techniques are well known and described for example in Sombrook et al (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

The present invention therefore also extends to a nucleic acid sequence, which encodes a mutant serum albumin according to the present invention.

For recombinant production of the mutant albumin in a host organism, the nucleotide sequence encoding the mutant albumin protein may be inserted into an expression cassette to form a DNA construct designed for a chosen host and introduced into the host where it is recombinantly produced. The choice of specific regulatory sequences such as promoter, signal sequence, 5′ and 3′ untranslated sequences, enhancer and terminator appropriate for the chosen host is within the level of skill of the routine worker in the art. The resultant molecule, containing the individual elements linked in a proper reading frame, may be introduced into the chosen cell using techniques well known to those in the art, such as calcium phosphate precipitation, electroporation, biolistic introduction,

virus introduction, etc. Suitable expression cassettes and vectors and methods for recombinant production of proteins are well known for host organisms such as E. coli (see eg. Studier and Moffatt, J. Mol. Biol. 189: 113 (1986); Brosius, DNA 8: 759 (1989)), yeast (see eg. Schneider and Guarente, Meth. Enzymol 194: 373 (1991) and insect cells (see eg. Luckow and Summers, Bio/Technol. 6: 47 (1988) and mammalian cell (tissue culture or gene therapy) by transfection (Schenborn ET, Goiffon V. Methods Mol Bio. 2000; 130: 135-45, Schenborn ET, Oler J. Methods Mol Biol. 2000; 130: 155-64), electroporation (Heiser WC. Methods Mol Biol. 2000; 130: 117-34), or recombinant viruses (Walther W. Stein U; Drugs 2000 Aug; 60 (2): 249-71).

Therefore, the invention further provides an expression cassette comprising a promoter operably linked to a nucleotide sequence as described herein encoding a mutant albumin as described herein. Nucleotide sequences encoding serum albumins, which may be mutated in accordance with the present invention, are also readily available from the Genbank database.

In addition, the invention provides a mutant albumin as described herein and a pharmaceutically acceptable carrier.

Pharmaceutically acceptable carriers are well known to those skilled in the art and include, but are not limited to, 0.1 M and preferably 0.05 M phosphate buffer or 0.8% saline. Additionally, such pharmaceutically acceptable carriers may be aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable

organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers such as those based on Ringer's dextrose, and the like. Preservatives and other additives may also be present, such as, for example, antimicrobials, antioxidants, chelating agents, inert gases and the like.

The mutant albumins of the present invention may be provided as pharmaceutical formulations wherein the mutant albumin is admixed with a pharmaceutically acceptable carrier (e.g. binder, corrective, corrigent, disintegrator, emulsion, excipient), diluent or solibilizer to give a pharmaceutical composition by a conventional manner, which is formulated into, for example, tablet, capsule, granule, powder, syrup, suspension, solution, injection, infusion, deposit agent, suppository and administered for example orally or parenterally.

When the tablets are used for oral administration, typically used carriers include sucrose, lactose, mannitol, maltitol, dextran, corn starch, typical lublicants such as magnesium stearate, preservatives such as paraben, sorbin, antioxidants such as ascorbic acid, α-tocopherol, cysteine, disintegrators or binders. When administered orally as capsules, effective diluents include lactose and dry corn starch. A liquid for oral use includes syrup, suspension, solution and emulsion, which may contain a typical inert diluent used in this field, such as water. In addition, sweeteners or flavours may be contained.

In the case of parenteral administration such as subcutaneous injection, intraveneous injection, intramuscular injection, intraperitoneal injection or infusion, the pH of the active ingredient solution may be appropriately adequately adjusted, bufferized or sterilized. Examples of usable vehicle or solvent include distilled water, Ringer water and isotonic brine. For intraveneous use, the total concentration of solute is adjusted to make the solution isotonic.

Suppositories may be prepared by admixing the compounds of the present invention with a suitable nonirritative excipient such as those that are solid at normal temperature but become liquid at the temperature in the intestine and melt in rectum to release the active ingredient, such as cocoa butter and polyethylene glycols.

The dose can be determined depending on age, body weight, administration time, administration method, combination of drugs, the level of condition for which a patient is undergoing therapy, and other factors. While the daily dose may vary depending on the conditions and body weight of patients, the species of active ingredient, and administration route, in the case of oral use, the daily dose may be about 0.1-100 mg/person/day, preferably 0.5-30 mg/person/day. In the case of parenteral use, the daily dose may desirably be 0.1-50 mg/person/day, preferably 0.1-30 mg/person/day for subcutaneous injection, intraveneous injection, intramuscular injection and intrarectal administration.

The mutant albumins of the present invention may be of use for example in human or animal medicine for the treatment of deficiency diseases and infections, treatment of metal overload and/or for conditions where control of metal concentrations may be linked to the physiological function of either another metal ion or an organic molecule, such as a drug or natural molecule. Another possible application is to introduce a catalytic site into albumin (as the site modeled looks like an enzyme site).

It may also be possible to regulate the amount of a metal, such as zinc, present in blood using the mutant albumins of the present invention, or facilitate treatment of a subject displaying problems with zinc absorption. Moreover, mutant albumins which display particularly strong metal binding affinity may be used in bisensors to detect metals in an environment.

Additionally observations that the Zinc bound to the albumin may be in the form of Zn^{2+} which may bind chloride ions, also leads to the possibility that albumin with bound zinc may be used as a chloride sensor and access to the Zn could be regulated by blood chloride concentration (this might also control catalytic acivity).

The present invention will now be further described by way of example and with reference to the figures, which show:

Figure 1 shows a model of the three dimension structure of human serum albumin as reported in PDB 1AO6, with the metal binding site identified herein, highlighted;

Figure 2 shows in more detail amino acid side-chains located in and around the proposed zinc binding site. Note that residue 248 is a glycine in the crystal structure (PDB 1AO6) but the albumin amino acid sequence states it to be a threonine residue;

Figure 3 shows an overlay between the original structure (black) and the present model (grey) when binding Zinc;

Figure 4 shows circular dichroism spectra of wild type (solid line), and H67A (dashed line) albumin.

Figure 5 shows ^{111}Cd NMR of native and H67A rHA with 2 mol equivalent of $^{111}CdCl_2$;

Figure 6 shows ^{111}Cd NMR of rHA with 2 mol equivalent of $^{111}CdCl_2$ in the presence of a) zinc and b) copper; and

Figure 7 shows UV-visible absorption spectra of (a) native rHA and (b) H67A rHA with 0.2 to 2 mol equivalent of $CuCl_2$ in 0.2 mol equivalent steps (bottom to top).

Materials and Methods

Computer programs and Databases

Sequence alignments were carried out using ClustalW, European Bioinformatics Institute (www.ebi.ac.uk/clustalw/) with sequences obtained from Entrez Protein, National Centre for Biotechnolgy Information (www.ncbi.nlm.nih.gov/entrez/). 3-dimensional

coordinates for human albumin (PDB 1AO6) were obtained from the Brookhaven Protein Databank (www.rcsb.org/pdb/).

Site-Directed Mutagenesis

Oligonucleotide-directed mutagenesis was used to prepare cDNAs encoding the H67A mutated form of albumin. The mutagenic oligonucleotides 5'-GCTGAAATTGTGACAAATCACTTGCTACCCTTTTTGGAGACAAATTATGC-3'and5'GCATAATTTGTCTCCAAAAAGGGTAGCAAGTGATTTGTCACAATTTTC AGC-3' were supplied by Delta Biotechnology Ltd., Nottingham. Mutagenesis was performed using the QuikChange® Site-Directed Mutagenesis Kit (Stratagene). A clone containing the desired mutation was identified by nucleotide sequence analysis across the mutation site by dideoxy chain termination sequencing. The mutated cDNA was inserted into a PUC9 yeast expression vector and transformed into *Saccharomyces cerevisiae* DXY1 cells by electroporation.

Expression and Purification

The *S. cerevisiae* DXY1 cell cultures, following growth at 30 °C for 4 days were centrifuged at 3,000 rpm for 30 min. The supernatants were then removed and filtered. The recombinant protein was concentrated from the supernatant, using cation-exchange chromatography. An SP-sepharose Fast Flow cation-exchange column (column volume = 225 mL) was equilibrated with 4 column volumes of a 30 mM sodium acetate buffer, pH 5.5. The filtered supernatants were split into two batches of approximately 3 L. Sodium octanoate was added (7.5 mL of a 2 M solution) to each batch and the pH was adjusted to 4.5 with acetic acid and the conductivity was adjusted to 5.5 mS cm^{-1} with

deionised water before loading onto the column. After loading, the column was then washed with 8 column volumes of 50 mM acetate, 8 mM NaOH, pH 4.0 and 4 column volumes of a 27 mM sodium acetate buffer containing 2 M NaCl, pH 4.0. A third wash was carried out with 10 column volumes of the equilibration buffer. Finally the column was eluted with 2 column volumes of 85 mM sodium acetate containing 5 mM octanoic acid, pH 5.5.

SP-sepharose Fast Flow eluents were then further purified by anion-exchange chromatography on a DEAE fast flow column (column volume = 167 mL). The column was equilibrated with 15 column volumes of 30 mM acetate, 27 mM NaOH, pH 5.5. The conductivity of SP-sepharose eluents was adjusted to 3.0 mS cm^{-1} with deionised water before loading onto the column. After loading the column was then washed with 5 column volumes of 15.7 mM $K_2B_4O_7 \cdot 4H_2O$, pH 9.2. The column was eluted with 0.75 column volumes of 85 mM acetate, 110 mM $K_2B_4O_7 \cdot 4H_2O$, pH 9.4..

DEAE eluents were then purified further by affinity chromatography on a Blue Agarose (Prometic Biosciences) column (column volume = 423 mL). Delta Blue matrix was supplied by ACL. The column was equilibrated with 2 column volumes of a 250 mM ammonium acetate buffer, pH 8.9 before loading the DEAE eluent. After loading, the column was then washed with 5 column volumes of the equilibration buffer. The column was eluted with 2 column volumes of 50 mM phosphate buffer containing 2 M NaCl, pH 6.9.

Delta Blue eluents were then concentrated using a 10 kD MWCO Pall Filtron LU Centramate filter connected to a peristaltic pump. It was determined that 4.25 g of H67A albumin was recovered. A sample of concentrated solution from the purified product was diluted to 5 mg mL^{-1} and 10 μL was applied to an SDS-PAGE gel. The gels were made and ran using the standard method of Laemmli (1970) Nature 227, 680-685. The gel was stained with both coomassie blue stain and silver stain and revealed no other proteins to be present at the 1% level (therefore protein is approximately 99% pure).

Circular Dichroism

Native recombinant human albumin (rHA) (Delta Biotechnology Ltd., Nottingham) and the H67A mutant albumins were diluted to approx. 1.5 mg mL^{-1} in 200 mM potassium phosphate, pH 7.4. Spectra were recorded for both of the proteins. The instrument used was a JASCO J-600 spectropolarimeter. Secondary structure estimations were calculated using the SELCON procedure.

^{111}Cd NMR Spectroscopy

^{111}Cd-NMR studies were carried out using 1.5 mM rHA or respective mutant protein at the same concentration, in 50 mM Tris, pH 7.1, 100 mM NaCl, 10% deuterium oxide with 2 mol equiv of $^{111}CdCl_2$. $^{111}CdCl_2$ was generated by dissolving ^{111}CdO (95.11% isotopic purity, Oak Ridge National Laboratory, Tennessee, USA) in the appropriate amount of 1 M HCl. Various equivalents of $ZnCl_2$ or $CuCl_2$ were added for metal titration experiments, the pH was checked and adjusted (if required) after each addition.

1D ^{111}Cd-{^{1}H} NMR spectra (106.04 MHz, Bruker DMX500) were acquired using a 10 mm BBO (direct observe) probe head. Proton decoupling was achieved by composite pulse decoupling using GARP. Typically, spectra were acquired over a sweep width of 30 kHz (280 ppm) into 4 k complex data points, with a ^{111}Cd pulse width of 17.5 μs (90°), 36 k transients, an acquisition time of 0.10 s, and a recycle delay of 0.30 s. Prior to Fourier Transformation, data were zero-filled to 16 k data points and apodized by exponential multiplication (120 Hz line broadening).

UV-Vis Spectrophotometry

Aliquots of $CuCl_2$ were added to 2 mM solutions of rHA and the H67A mutant in 200 mM potassium phosphate, pH 7.4. Spectra were recorded using a Shimadzu UV250 1PC spectrophotometer between 400 to 800 nm.

Examples

Identification of Zinc Binding Site by Molecular Modelling

NMR studies have revealed that ^{113}Cd chemical shifts upon binding to albumin suggest metal coordination to the protein at 2 sites. (Sadler and Viles (1996) Inorg. Chem. 35, 4490-4496). At the site where Zn^{2+} displaces Cd^{2+} the chemical shift is in the range for coordination of the metal to the protein to involve 2–3 imidazole nitrogens (Öz *et al.* (1998) Biochem. Cell Biol. 76, 223-234).

The crystal structure coordinates of human albumin were obtained from the Brookhaven Protein Databank (PDB 1AO6) and were examined using WebLab Viewer Pro v4.0

(Accelrys). Histidine residues were highlighted (since these are the main nitrogen donating residues in proteins for metal coordination) and distances between each were measured. The present inventors found that only one site on the molecule had present 2 histidine side-chains within 5 Å from each other. This led us to believe that His67 and His247 were involved in the zinc binding site. The identification of other residues around this site revealed that Asn99 and Asp249 were also within close enough proximity to provide oxygen ligands for metal binding. Asn99 could also potentially provide a nitrogen ligand from the amide group of its side chain.

A database (Harding (2001) Acta Cyst. D57, 401-411; http://tanna.bch.ed.ac.uk) of amino acid side-chains coordinating to metals in proteins revealed that 3 other proteins contain zinc bound to 2 His, 1 Asp and 1 Asn residues (human calcineurin, *E. coli* 5'-endonucleotidase and kidney bean purple phosphatase) further suggesting this to be a suitable site for zinc binding. See Figures 1 and 2, which show the predicted region of metal binding as determined by the present inventors.

Modelling of Zinc into Proposed Binding Site

An initial model of Zn-containing albumin was built based on the published crystal structure (pdb accession code 1AO6) using Weblabviewer (Accelrys). The zinc site was modelled as 5 coordinate containing Cl^- as the fifth ligand, since in our 1D ^{111}Cd NMR studies we have noticed that the shift of the resonance is dependent on the Cl^- concentration, which makes binding of chloride under physiological conditions highly likely. Water as a fifth ligand is another possibility.

The model was imported into Sybyl v6.8 (TRIPOS Inc.) for energy minimization to optimise geometry, using the TRIPOS force field, after some specific parameters for zinc had been defined. Bond lengths for Zn^{2+} bound to histidine (2.00 Å) and aspartate (2.00 Å) (and water, 2.06 Å) were taken from Harding (2001) Acta Cyst. D57, 401-411, and a bond length for an Asn-Zn^{2+} interaction (2.15 Å) was estimated based on the crystal structures of calcineurin, 5'-endonucleotidase and kidney bean purple acid phosphatase, which were obtained from the Brookhaven Protein Databank (pdb accession codes 4KPB, 1AUI and 1TCO). The value for the Zn-Cl bond length was extracted from the Cambridge structural database (Allen and Kennard (1993) Chem. Design Autom. News 8, 31-37). Force constants were taken from the TRIPOS force field. Bond angles around zinc were not constrained at all, because for Zn^{2+} with a coordination number of 5, no regular or uniform angles are to be expected.

In a first step, the geometry around the zinc was optimised by 200 steps of energy minimisation of the zinc atom, the four protein ligand residues, and the chloride ion only. A further 10 steps of energy minimisation were then employed on the entire protein to remove bad geometries and Van der Waals contacts which had been introduced through the atom movements in the first step. The root mean square deviation (rmsd) values (which are an indication of structural difference) between the original protein structure and the modified model is 0.13 Å for all atoms, and 1.21 Å for the ligands residue atoms only.

Figure 3 shows an overlay between the original structure (black) without hydrogens) and the present inventors model (grey) demonstrating that only relatively small movements

were necessary to accommodate the zinc binding site. The site displays a distorted trigonal bipyramidal geometry with the two histidines in the axial positions. The chloride ligand points towards the outside of the protein. Additional modelling attempts with different starting structures furnished sites with similar geometries, but with the chloride ion on the opposite side of the Zn.

Attempts to model a tetrahedral site containing only the protein ligands yielded, despite applying angle constraints, a geometry resembling the distorted trigonal bipyramid found in the 5-coordinate model, with an empty equatorial binding site where the Cl^- had been.

Experimental evidence to support modeling theories

The present inventors expressed the mutant H67A in *Saccharomyces cerevisiae* DXY1 cells and purified it to >95% by ion exchange and affinity chromatography. Circular dichroism revealed no major alterations in secondary structure between the H67A mutant and the wild-type protein (Figure 4). ^{111}Cd-NMR studies on 1.5 mM recombinant human albumin (rHA), in 50 mM Tris, pH 7.1 with 2 mol equiv of $^{111}CdCl_2$ confirmed binding at 2 sites (A and B) with peaks at 27 and 131 ppm (relative to $Cd(ClO_4)$), respectively. Under the same conditions the H67A mutant gave rise to a single peak at 29 ppm (Figure 5). Addition of 0.5 and 1 mol equiv of $ZnCl_2$ to rHA in the presence of 2 mol equiv of $^{111}Cd^{2+}$ resulted in a decrease in intensity of the peak at 131 ppm (Figure 6a). Addition of 2 and 3 mol equiv of $CuCl_2$ to rHA in the presence of 2 mol equiv of $^{111}CdCl_2$ also appeared to affect Cd^{2+} binding at site B and led to the formation of a new ^{111}Cd peak at 37 ppm (Figure 6b). The addition of 1 mol equiv of $CuCl_2$ did not affect Cd^{2+} binding. This is most likely due to the high affinity of Cu^{2+} for the N-terminus, with Cd^{2+}

displacement occurring only after saturation of binding at this site. These results show that site B has a greater affinity toward Zn^{2+} than to Cd^{2+}, that Cu^{2+} also binds competitively at this site, and also suggest the involvement of His67 for metal coordination.

Note also that figure 4 shows similar signs and magnitudes of circular dichroism bands for native, and H67A. This is indicative of H67A having similar secondary structure to native albumin.

The number of nitrogen ligands coordinating to Cu^{2+} in peptides and proteins is known to affect the wavelength of the d-d absorption bands of these complexes. Aliquots of $CuCl_2$ were added to 2 mM solutions of rHA and the H67A mutant in 200 mM potassium phosphate, pH 7.4. An absorption band at 525 nm appeared after the first addition of $CuCl_2$, indicative of N-terminal loading of the proteins by Cu^{2+}, characteristic of 4 N coordination to Cu^{2+}. However a marked difference in absorption was observed after the further addition of 1 mol equiv $CuCl_2$ to each of the proteins. The native protein developed a second absorption band at 625 nm and the mutant a much broader band at 750 nm (Figure 7). These bands suggest coordination of Cu^{2+} to 2 N and 1 N respectively (Pettit *et al.* (1990) *J. Chem. Soc. Dalton. Trans.* 3565-3570). This suggests that the His67 residue is important for Cu^{2+} binding as well as Zn^{2+}, although does not provide information as to whether the Cu^{2+} ions still bind at this site (without the involvement of His67) or elsewhere on the protein.

As a result of theses studies it is possible to prepare novel mutant albumins with decreased or enhanced affinities for metal ions such as Zn^{2+} by mutation of residues around the locus of the metal site. These include mutation of a side-chain, which can bind metals to one which cannot (or only weakly) bind is likely to give decreased metal affinity.

```
HUMAN       MKWVTFISLLFLFSSAYSRGVFRRDAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQCPF 60
MACAQUE     --------LLFLFSSAYSRGVFRRDTHKSEVAHRFKDLGEEHFKGLVLVAFSQYLQQCPF 52
CANINE      MKWVTFISLFFLFSSAYSRGLVRREAYKSEIAHRYNDLGEEHFRGLVLVAFSQYLQQCPF 60
FELINE      MKWVTFISLLLLFSSAYSRGVTRREAHQSEIAHRFNDLGEEHFRGLVLVAFSQYLQQCPF 60
BOVINE      MKWVTFISLLLLFSSAYSRGVFRRDTHKSEIAHRFKDLGEEHFKGLVLIAFSQYLQQCPF 60
SHEEP       MKWVTFISLLLLFSSAYSRGVFRRDTHKSEIAHRFNDLGEENFQGLVLIAFSQYLQQCPF 60
PIG         --WVTFISLLFLFSSAYSRGVFRRDTYKSEIAHRFKDLGEQYFKGLVLIAFSQHLQQCPY 58
RABBIT      MKWVTFISLLFLFSSAYSRGVFRREAHKSEIAHRFNDVGEEHFIGLVLITFSQYLQKCPY 60
RAT         MKWVTFLLLLFISGSAFSRGVFRREAHKSEIAHRFKDLGEQHFKGLVLIAFSQYLQKCPY 60
                 .  :   *::: .**:*** **::::**:***::*:**: * .***::*:*:**:**:

HUMAN       EDHVKLVNEVTEFAKTCVADESAENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEP 120
MACAQUE     EEHVKLVNEVTEFAKTCVADESAENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEP 112
CANINE      EDHVKLAKEVTEFAKACAAEESGANCDKSLHTLFGDKLCTVASLRDKYGDMADCCEKQEP 120
FELINE      EDHVKLVNEVTEFAKGCVADQSAANCEKSLHELLGDKLCTVASLRDKYGEMADCCEKKEP 120
BOVINE      DEHVKLVNELTEFAKTCVADESHAGCEKSLHTLFGDELCKVASLRETYGDMADCCEKQEP 120
SHEEP       DEHVKLVKELTEFAKTCVADESHAGCDKSLHTLFGDELCKVATLRETYGDMADCCEKQEP 120
PIG         EEHVKLVREVTEFAKTCVADESAENCDKSIHTLFGDKLCAIPSLREHYGDLADCCEKEEP 118
RABBIT      EEHAKLVKEVTDLAKACVADESAANCDKSLHDIFGDKICALPSLRDTYGDVADCCEKKEP 120
RAT         EEHIKLVQEVTDFAKTCVADENAENCDKSIHTLFGDKLCAIPKLRDNYGELADCCAKQEP 120
            ::* **..*:*::** *.*::.  .*:**:* ::**::* :..**: **:**** *:**

HUMAN       ERNECFLQHKDDNPNLPRLVRPEVDVMCTAFHDNEETFLKKYLYEIARRHPYFYAPELLF 180
MACAQUE     ERNECFLQHKDDNPNLPPLVRPEVDVMCTAFHDNEATFLKKYLYEVARRHPYFYAPELLF 172
CANINE      DRNECFLAHKDDNPGFPPLVAPEPDALCAAFQDNEQLFLGKYLYEIARRHPYFYAPELLY 180
FELINE      ERNECFLQHKDDNPGFGQLVTPEADAMCTAFHENEQRFLGKYLYEIARRHPYFYAPELLY 180
BOVINE      ERNECFLSHKDDSPDLPKLK-PDPNTLCDEFKADEKKFWGKYLYEIARRHPYFYAPELLY 179
SHEEP       ERNECFLNHKDDSPDLPKLK-PEPDTLCAEFKADEKKFWGKYLYEVARRHPYFYAPELLY 179
PIG         ERNECFLQHKNDNPDIPKLK-PDPVALCADFQEDEQKFWGKYLYEIARRHPYFYAPELLY 177
RABBIT      ERNECFLHHKDDKPDLPPFARPEADVLCKAFHDDEKAFFGHYLYEVARRHPYFYAPELLY 180
RAT         ERNECFLQHKDDNPNLPPFQRPEAEAMCTSFQENPTSFLGHYLHEVARRHPYFYAPELLY 180
            :****** **:*.*.:  :  *:  .:*  *: :   *  :**:*:************:

HUMAN       FAKRYKAAFTECCQAADKAACLLPKLDELRDEGKASSAKQRLKCASLQKFGERAFKAWAV 240
MACAQUE     FAARYKAAFAECCQAADKAACLLPKLDELRDEGKASSAKQRLKCASLQKFGDRAFKAWAV 232
CANINE      YAQQYKGVFAECCQAADKAACLGPKIEALREKVLLSSAKERFKCASLQKFGDRAFKAWSV 240
FELINE      YAEEYKGVFTECCEAADKAACLTPKVDALREKVLASSAKERLKCASLQKFGERAFKAWSV 240
BOVINE      YANKYNGVFQECCQAEDKGACLLPKIETMREKVLASSARQRLRCASIQKFGERALKAWSV 239
SHEEP       YANKYNGVFQECCQAEDKGACLLPKIDAMREKVLASSARQRLRCASIQKFGERALKAWSV 239
PIG         YAIIYKDVFSECCQAADKAACLLPKIEHLREKVLTSAAKQRLKCASIQKFGERAFKAWSL 237
RABBIT      YAQKYKAILTECCEAADKGACLTPKLDALEGKSLISAAQERLRCASIQKFGDRAYKAWAL 240
RAT         YAEKYNEVLTQCCTESDKAACLTPKLDAVKEKALVAAVRQRMKCSSMQRFGERAFKAWAV 240
            :*  *:  : :**   **.*** **:: :. :   ::.::*::*:*:*:**:** ***::

HUMAN       ARLSQRFPKAEFAEVSKLVTDLTKVHTECCHGDLLECADDRADLAKYICENQDSISSKLK 300
MACAQUE     ARLSQKFPKAEFAEVSKLVTDLTKVHTECCHGDLLECADDRADLAKYMCENQDSISSKLK 292
CANINE      ARLSQRFPKADFAEISKVVTDLTKVHKECCHGDLLECADDRADLAKYMCENQDSISTKLK 300
FELINE      ARLSQKFPKAEFAEISKLVTDLAKIHKECCHGDLLECADDRADLAKYICENQDSISTKLK 300
BOVINE      ARLSQKFPKAEFVEVTKLVTDLTKVHKECCHGDLLECADDRADLAKYICDNQDTISSKLK 299
SHEEP       ARLSQKFPKADFTDVTKIVTDLTKVHKECCHGDLLECADDRADLAKYICDHQDALSSKLK 299
PIG         ARLSQRFPKADFTEISKIVTDLAKVHKECCHGDLLECADDRADLAKYICENQDTISTKLK 297
RABBIT      VRLSQRFPKADFTDISKIVTDLTKVHKECCHGDLLECADDRADLAKYMCEHQETISSHLK 300
RAT         ARMSQRFPNAEFAEITKLATDVTKINKECCHGDLLECADDRAELAKYMCENQATISSKLQ 300
            .*:**:**:*:*.:::*:.**::*::.****************:****:*::* ::*::*:

HUMAN       ECCEKPLLEKSHCIAEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLGMFLYEYAR 360
MACAQUE     ECCDKPLLEKSHCLAEVENDEMPADLPSLAADYVESKDVCKNYAEAKDVFLGMFLYEYAR 352
CANINE      ECCDKPVLEKSQCLAEVERDELPGDLPSLAADFVEDKEVCKNYQEAKDVFLGTFLYEYSR 360
FELINE      ECCGKPVLEKSHCISEVERDELPADLPPLAVDFVEDKEVCKNYQEAKDVFLGTFLYEYSR 360
BOVINE      ECCDKPLLEKSHCIAEVEKDAIPENLPPLTADFAEDKDVCKNYQEAKDAFLGSFLYEYSR 359
SHEEP       ECCDKPVLEKSHCIAEVDKDAVPENLPPLTADFAEDKEVCKNYQEAKDVFLGSFLYEYSR 359
PIG         ECCDKPLLEKSHCIAEAKRDELPADLNPLEHDFVEDKEVCKNYKEAKDVFLGTFLYEYSR 357
RABBIT      ECCDKPILEKAHCIYGLHNDEDTAGLPAVAEEFVEDKDVCKNYEEAKDLFLGKFLYEYSR 360
RAT         ACCDKPVLQKSQCLAETEHDNIPADLPSIAADFVEDKEVCKNYAEAKDVFLGTFLYEYSR 360
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HUMAN      RHPDYSVVLLLRLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQNLIKQNCELFE 420
MACAQUE    RHPDYSVMLLLRLAKAYEATLEKCCAAADPHECYAKVFDEFQPLVEEPQNLVKQNCELFE 412
CANINE     RHPEYSVSLLLRLAKEYEATLEKCCATDDPPTCYAKVLDEFKPLVDEPQNLVKTNCELFE 420
FELINE     RHPEYSVSLLLRLAKEYEATLEKCCATDDPPACYAHVFDEFKPLVEEPHNLVKTNCELFE 420
BOVINE     RHPEYAVSVLLRLAKEYEATLEECCAKDDPHACYSTVFDKLKHLVDEPQNLIKQNCDQFE 419
SHEEP      RHPEYAVSVLLRLAKEYEATLEDCCAKEDPHACYATVFDKLKHLVDEPQNLIKKNCELFE 419
PIG        RHPDYSVSLLLRIAKIYEATLEDCCAKEDPPACYATVFDKFQPLVDEPKNLIKQNCELFE 417
RABBIT     RHPDYSVVLLLRLGKAYEATLKKCCATDDPHACYAKVLDEFQPLVDEPKNLVKQNCELYE 420
RAT        RHPDYSVSLLLRLAKKYEATLEKCCAEGDPPACYGTVLAEFQPLVEEPKNLVKTNCELYE 420
           ***:*:* :***:.* **:**:.***  **  **. *: ::: **:**:**:* **: :*

HUMAN      QLGEYKFQNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAKRMPCAEDYLSVV 480
MACAQUE    QLGEYKFQNALLVRYTKKVPQVSTPTLVEVSRNLGKVGAKCCKLPEAKRMPCAEDYLSVV 472
CANINE     KLGEYGFQNALLVRYTKKAPQVSTPTLVEVSRKLGKVGTKCCKKPESERMSCADDFLSVV 480
FELINE     KLGEYGFQNALLVRYTKKVPQVSTPTLVEVSRSLGKVGSKCCTHPEAERLSCAEDYLSVV 480
BOVINE     KLGEYGFQNALIVRYTRKVPQVSTPTLVEVSRSLGKVGTRCCTKPESERMPCTEDYLSLI 479
SHEEP      KLGEYGFQNALIVRYTRKAPQVSTPTLVEISRSLGKVGTKCCAKPESERMPCTEDYLSLI 479
PIG        KLGEYGFQNALIVRYTKKVPQVSTPTLVEVARKLGLVGSRCCKRPEEERLSCAEDYLSLV 477
RABBIT     QLGDYNFQNALLVRYTKKVPQVSTPTLVEISRSLGKVGSKCCKHPEAERLPCVEDYLSVV 480
RAT        KLGEYGFQNAVLVRYTQKAPQVSTPTLVEAARNLGRVGTKCCTLPEAQRLPCVEDYLSAI 480
           : *:* ****::****:*.********** :*.** **::**  ** :*:.*.:*:** :

HUMAN      LNQLCVLHEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFNAETFTFHADICTL 540
MACAQUE    LNRLCVLHEKTPVSEKVTKCCTESLVNRRPCFSALELDEAYVPKAFNAETFTFHADMCTL 532
CANINE     LNRLCVLHEKTPVSERVTKCCSESLVNRRPCFSGLEVDETYVPKEFNAETFTFHADLCTL 540
FELINE     LNRLCVLHEKTPVSERVTKCCTESLVNRRPCFSALQVDETYVPKEFSAETFTFHADLCTL 540
BOVINE     LNRLCVLHEKTPVSEKVTKCCTESLVNRRPCFSALTPDETYVPKAFDEKLFTFHADICTL 539
SHEEP      LNRLCVLHEKTPVSEKVTKCCTESLVNRRPCFSDLTLDETYVPKPFDEKFFTFHADICTL 539
PIG        LNRLCVLHEKTPVSEKVTKCCTESLVNRRPCFSALTPDETYKPKEFVEGTFTFHADLCTL 537
RABBIT     LNRLCVLHEKTPVSEKVTKCCSESLSNRRPCFSALGPDETYVPKEFNAETFTFHADICTL 540
RAT        LNRLCVLHEKTPVSEKVTKCCSGSLVERRPCFSALTVDETYVPKEFKAETFTFHSDICTL 540
           **:***********::*****: ** :****** *  **:* ** *    ****:*:***

HUMAN      SEKERQIKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCCKADDKETCFAEEGKKLV 600
MACAQUE    SEKEKQVKKQTALVELVKHKPKATKEQLKGVMDNFAAFVEKCCKADDKEACFAEEGPKFV 592
CANINE     PEAEKQVKKQTALVELLKHKPKATDEQLKTVMGDFGAFVEKCCAAENKEGCFSEEGPKLV 600
FELINE     PEAEKQIKKQSALVELLKHKPKATEEQLKTVMGDFGSFVDKCCAAEDKEACFAEEGPKLV 600
BOVINE     PDTEKQIKKQTALVELLKHKPKATEEQLKTVMENFVAFVDKCCAADDKEACFAVEGPKLV 599
SHEEP      PDTEKQIKKQTALVELLKHKPKATDEQLKTVMENFVAFVDKCCAADDKEGCFVLEGPKLV 599
PIG        PEDEKQIKKQTALVELLKHKPHATEEQLRTVLGNFAAFVQKCCAAPDHEACFAVEGPKFV 597
RABBIT     PETERKIKKQTALVELVKHKPHATNDQLKTVVGEFTALLDKCCSAEDKEACFAVEGPKLV 600
RAT        PDKEKQIKKQTALAELVKHKPKATEDQLKTVMGDFAQFVDKCCKAADKDNCFATEGPNLV 600
           .: *:::***:**.**:****:**.:**: *: :*  :::*** * ::: **  ** ::*

HUMAN      AASQAALGL 609
MACAQUE    AASQAALA- 600
CANINE     AAAQAALV- 608
FELINE     AAAQAALA- 608
BOVINE     VSTQTALA- 607
SHEEP      ASTQAALA- 607
PIG        IEIRGILA- 605
RABBIT     ESSKATLG- 608
RAT        ARSKEALA- 608
              :  *
```

Table 1. Comparison of amino acid sequence between mammalian albumins. Residues, in the locus of the zinc binding site are highlighted. Amino acids before the N terminal amino acid (residue number 1), in the boxed area, are part of the pre-albumin sequence and are cleaved following translation to give albumin itself. Accession numbers of the sequences are Human, P02768; Macaque,M90463; Canine, CAB64867; Feline, P49064; Bovine,P02769; Sheep, P14639; Pig, ABPGS; Rabbit, P49065 and Rat, P02770.

Figure 1

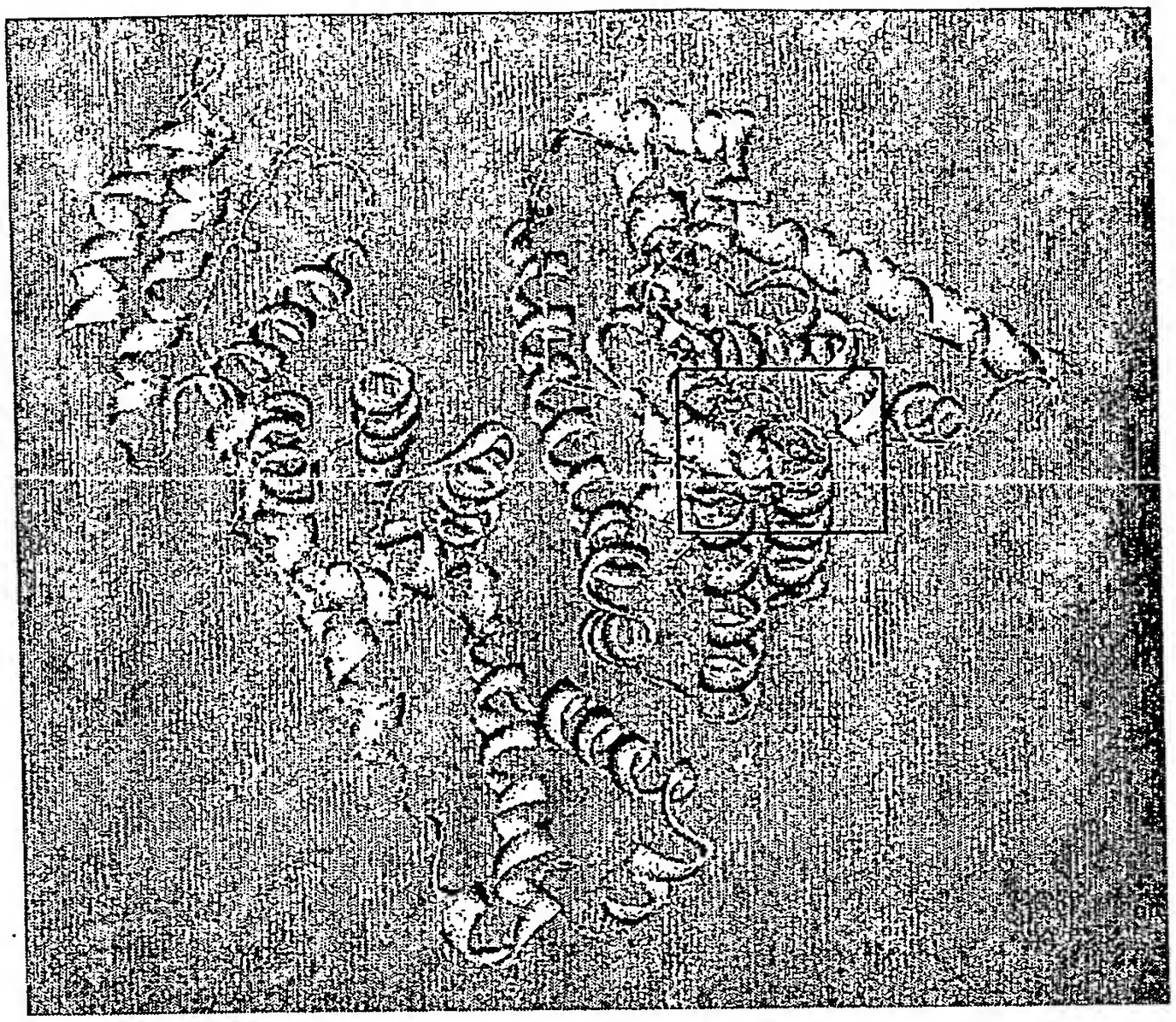


Figure 2

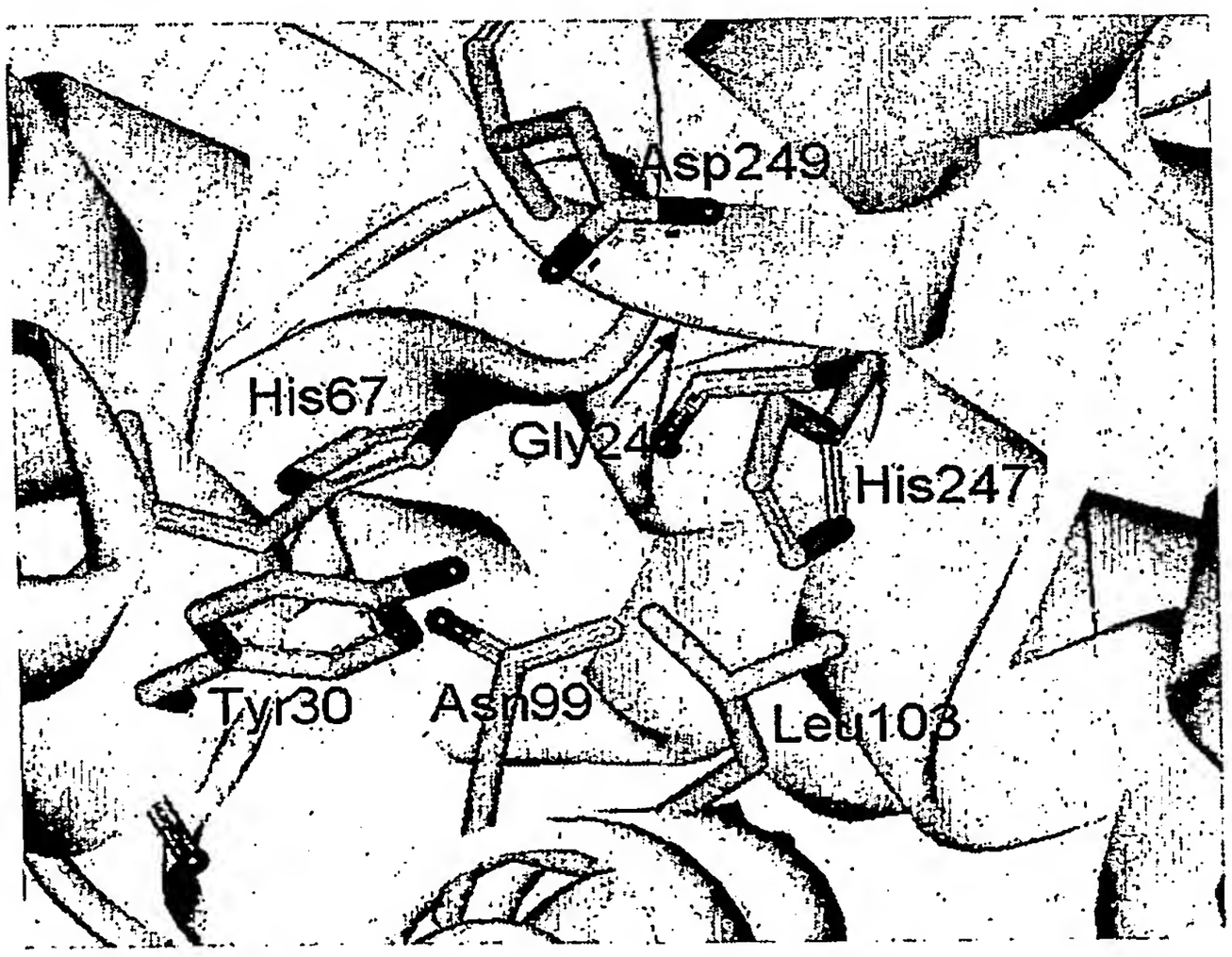


Figure 3

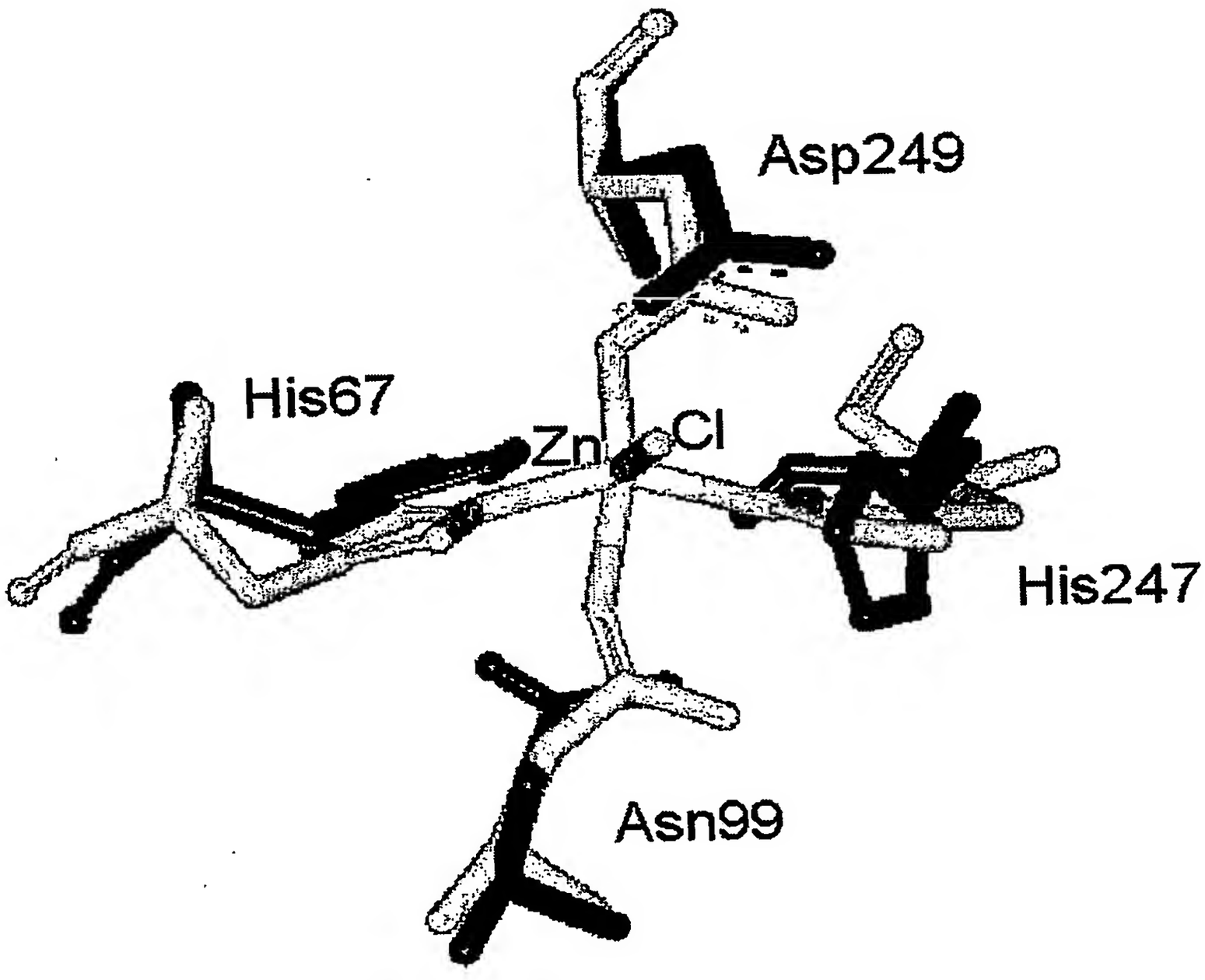
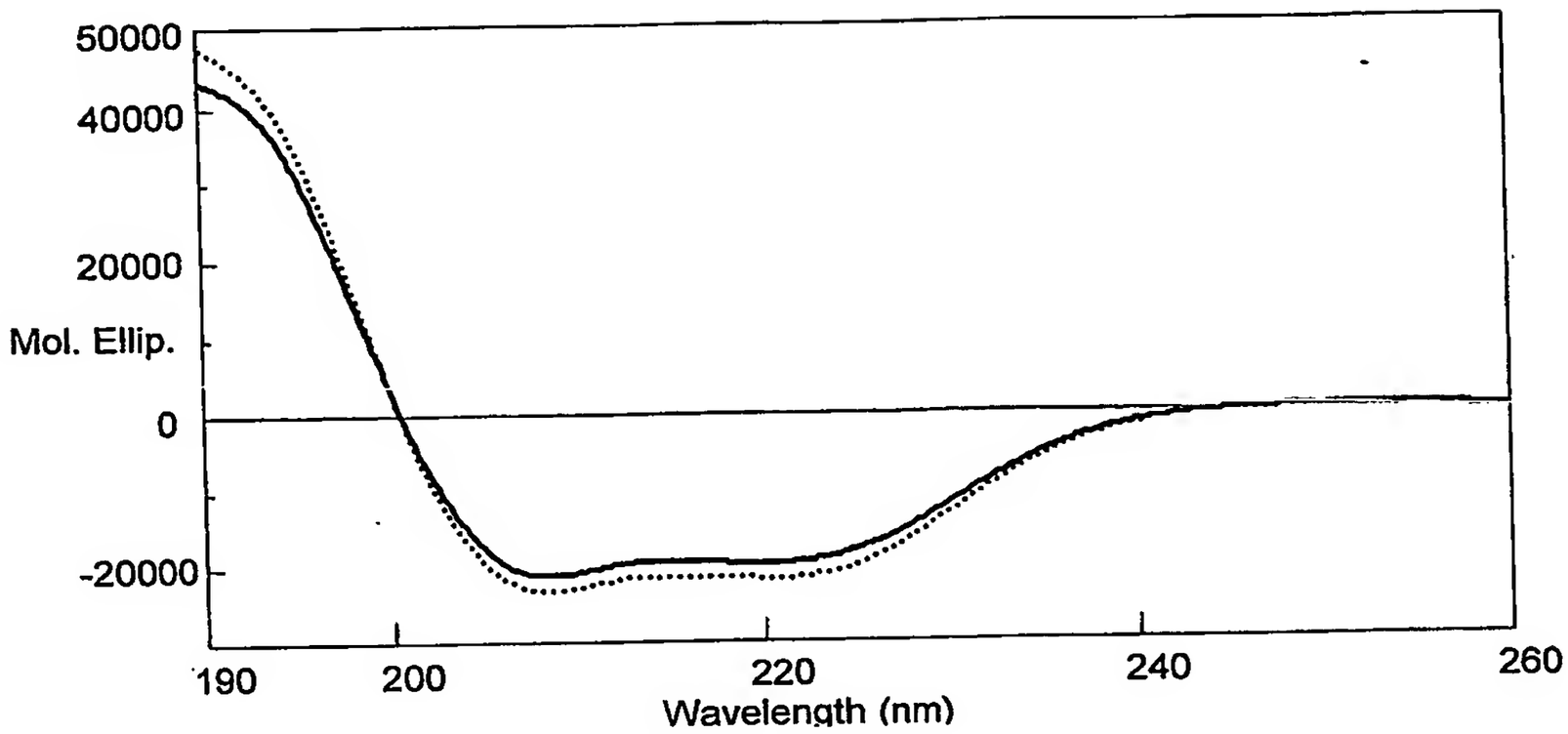


Figure 4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Mol. Ellip.
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-20000
190
200
220
240
260
Wavelength (nm)

Figure 5

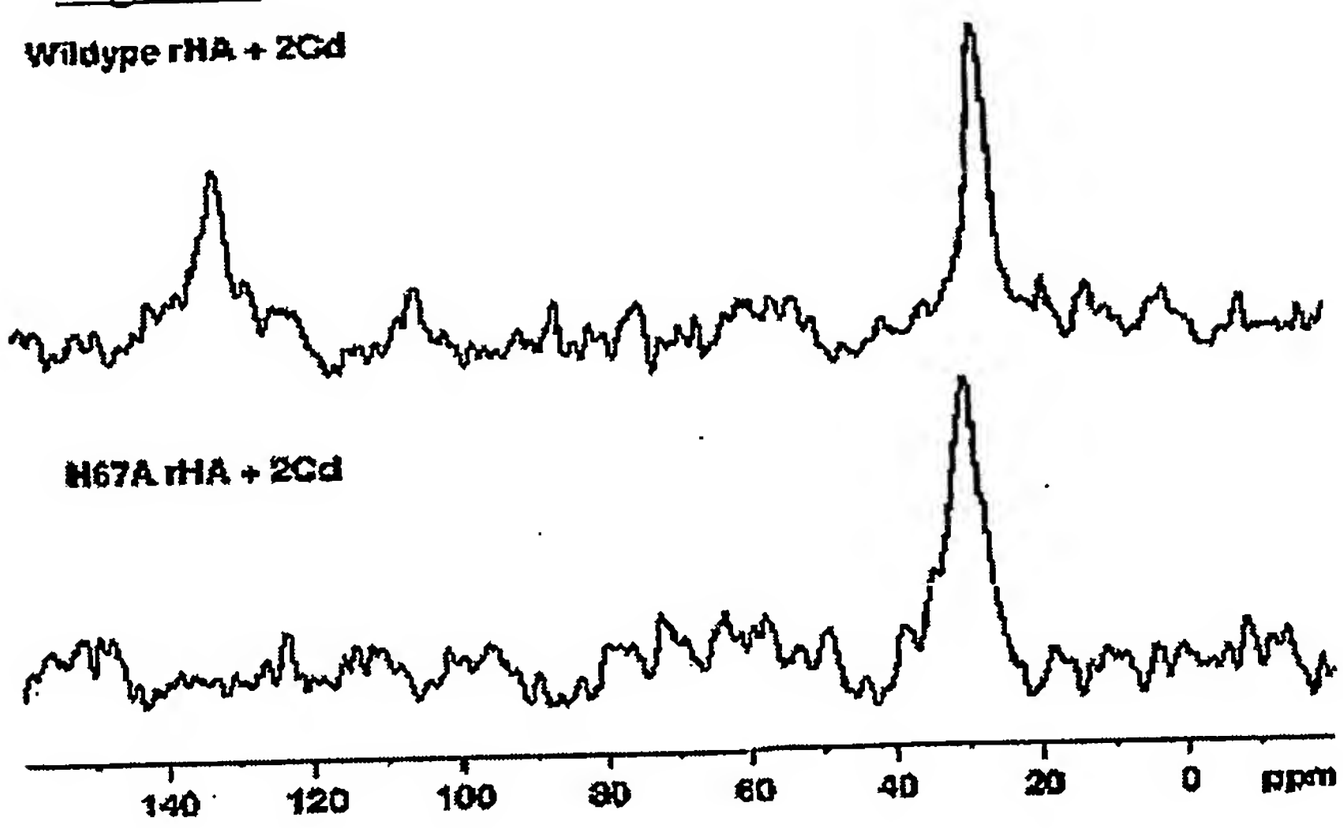
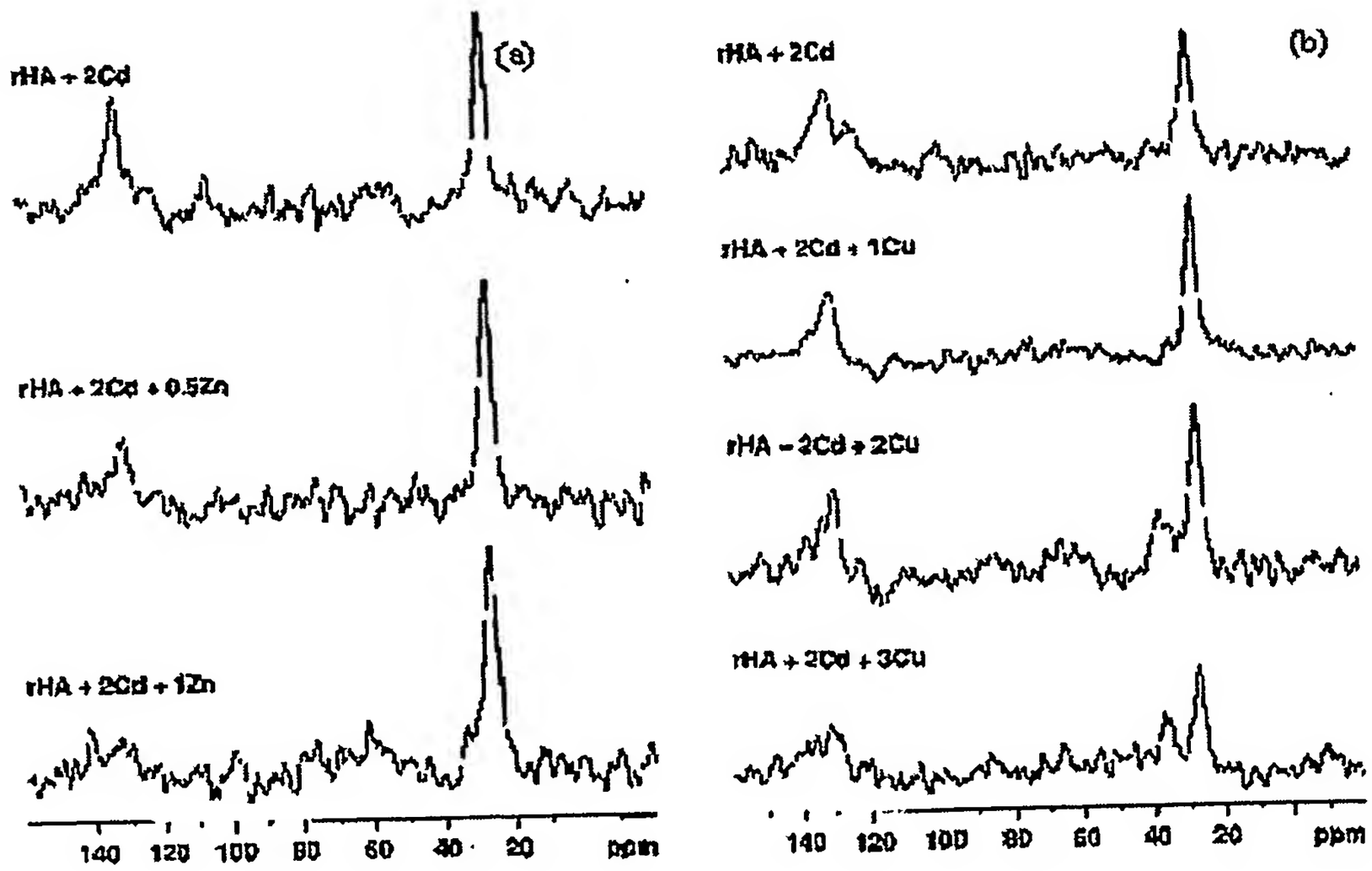
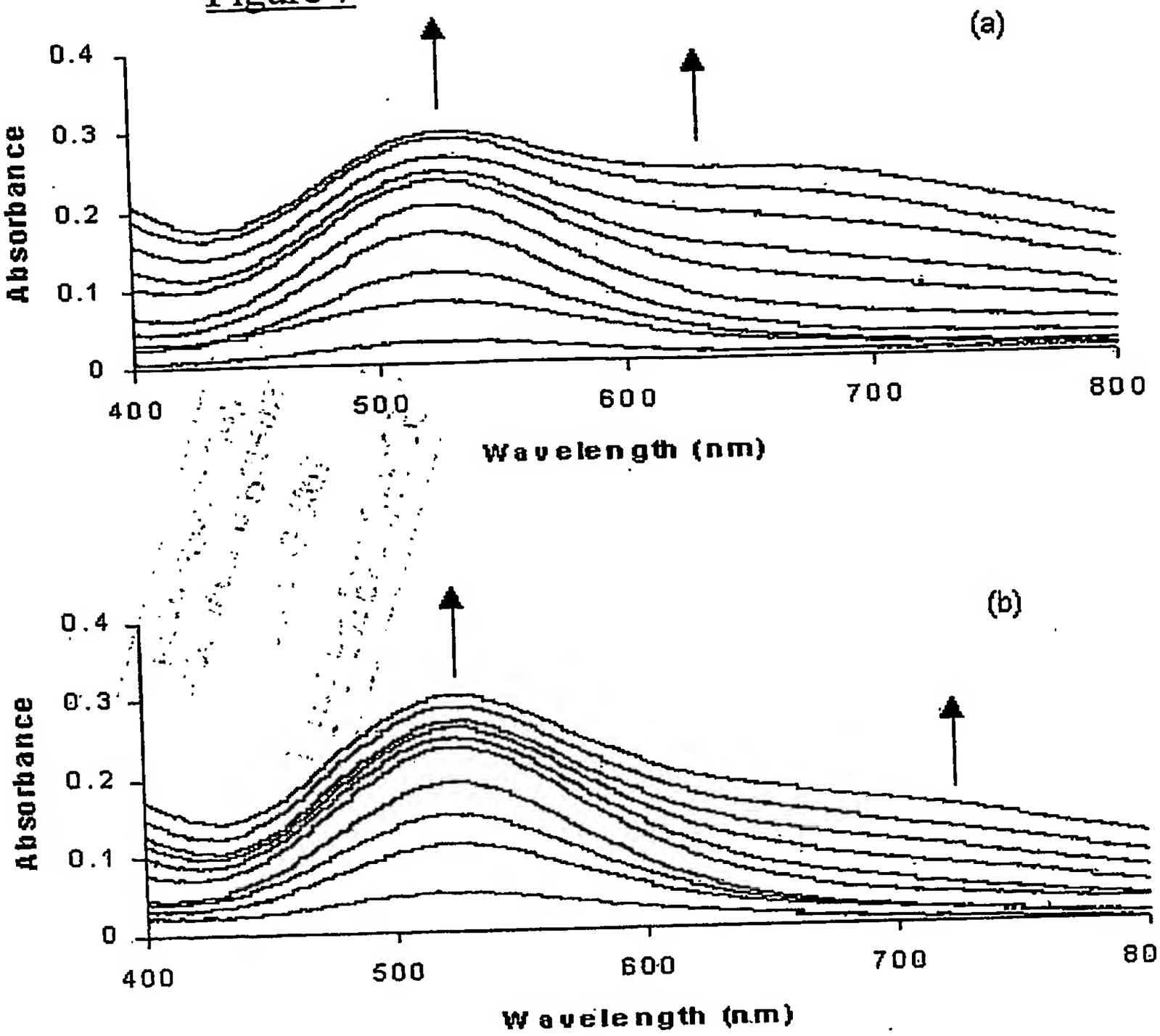


Figure 6



02173[redacted]7.4

Figure 7


(a)
Absorbance
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0.3
0.2
0.1
0
400
500
600
700
800
Wavelength (nm)
(b)
Absorbance
0.4
0.3
0.2
0.1
0
400
500
600
700
800
Wavelength (nm)

PCT GB